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EFFECT OF WEIGHTLESSNESS CONDITIONS ON THE SOMATIC EMBRYOGENESIS IN THE CULTURE OF CARROT CELLS

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Translation of "Vliyaniye usloviy nevesomosti na somaticheskiy embriogenez v kul'ture kletok morkovi," USSR Academy of Sciences, Moscow, Report, 1977, pp. 1-18

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16. Abstract A carrot cell culture seeded in Petri dishes in the United States and transported to the USSR was then subjected to weightlessness for 20 days during the flight of Kosmos 782. The controls were cultures placed on a centrifuge (1 g) inside the satellite and cultures left on ground in the USSR and the United States. A count of structures in the dishes after the flight showed that the number of developing embryonic structures and the extent of their differentiation in weightlessness did not reliably differ from the number and extent of differentiation in structures developed on the ground. Structures with long roots developed in weightlessness. Analysis of the root zones showed that these roots differed by the increased size of the zone of differentiated cells. The increased size of the zones of differentiated cells can indicate earlier development of embryonic structures.

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EFFECT OF WEIGHTLESSNESS CONDITIONS ON THE SOMATIC EMBRYOGENESIS IN THE CULTURE OF CARROT CELLS

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A culture of isolated plant cells is a convenient model for /1* investigating aspects of the vital activity of plant cells. This model affords a new way of studying the nutrition of a cell, its oxygen supply, and the cell's response to chemical factors, including hormonal. Much less studied is the response of cell cultivation to physical factors. Among the latter, gravity is doubtless of interest, a factor whose influence at the level of the intact plant and plant organs has been investigated for more than a hundred years now.

This study deals with the effect of the absence of gravity (or weightlessness) on embryogenesis in cultures of isolated cells. Steward /1/, Butenko /2/, and other authors /3,4/ demonstrated that somatic cells of plants in certain conditions of in vitro culturing can give rise to embryonic structures, which as a result of development form a normal embryo, and then an intact plant. Since it was shown /5/ that, beginning with certain stages, somatic embryogenesis is absolutely identical to sexual embryogenesis, this model was found very convenient in studying aspects of embryogenesis and the effect of external factors on embryogenesis.

In Steward's laboratory in the United States /6/, an experimental model was elaborated in detail based on carrot cell cultures; the model permits experiments with seeding in an agarized medium a large number of isolated cells capable of embryogenesis. /2

* Numbers in margin indicate pagination in the foreign text.

apart from the intact organism, in strictly controlled conditions and in a period convenient for the experimenter.

These advantages of the model developed made it very convenient for space experiments.

Materials and Methods

A culture of carrot cells prepared in Dr. A. Krikorian's laboratory in New York University, Stony Brook (US) was the study material. This culture was first prepared with explants from wild carrot seedlings and then grown in a suspension culture in White's medium enriched with coconut milk (10 percent), naphthylacetic acid /NAA/ (2 mg/liter), and casein hydrolysate (0.2 percent). In certain conditions, cells seeded in the agarized medium form in 21 days embryonic structures at different developmental stages.

The culture was filtered at Stony Brook through a system of filters; as a result, the suspension now consisted of individual cells and small clusters--their size was not more than 74 μ. The suspension was transferred to Murasig-Skug's medium, devoid of coconut milk and NAA, but containing inositol (20 mg/liter) and 3 percent saccharose. The cell suspension was blended with the agarized nutrient medium, cooled to 48° C, and then rapidly developed in Petri dishes. Now the culture prepared contained about 40,000 cells and small clusters. Special 50 mm diameter dishes with "clips"--for hermeticity--were used. Dishes housing the seeded culture were secured in groups of nine in 14 special containers.

Two containers were installed inside the satellite. One /3
was on the centrifuge during the flight, providing 1 g and simulating Earth gravity. The second was in weightlessness.

The conditions inside the satellite provided the following:

1. temperature: 18.3-23.2° C
2. relative humidity: 40-63 percent
3. O_2 pressure: 126-253 mm Hg
4. CO_2 pressure: 3-4.5 mm Hg

The satellite experiment lasted 20 days.

For electron microscopy in No. 3 dishes, from each container 1 percent glutaraldehyde prepared in 0.05 M phosphate buffer was the fixative, for 2 h; then, the 1 percent glutaraldehyde was replaced with a 3 percent glutaraldehyde solution prepared in the same buffer. The material was kept in this solution at 2° C.

Fixation after Brodskiy and Carnua in dishes Nos. 7 and 9, respectively, was conducted for cytological examination.

The Brodskiy fixative contained: 4 percent formalin (ten parts), 96 percent ethanol (three parts), glacial acetic acid (one part); Carnua's fixative contained: glacial acetic acid (one part), chloroform (three parts), and 96 percent ethanol (six parts).

The material was fixed for 2 h, then the fixative was replaced with 96 percent ethanol (24 h), then transferred to 70 percent ethanol, in which the material was kept at 2° C.

The structures were counted in three square cm of agarized medium cut from each dish. To determine the number of structures /4 per dish, the number of structures found in 1 cm^2 was multiplied by a coefficient of 19.6. At the same time as the structures were being counted, the total length of the embryos, and the length of roots and the "above-ground" part of the seedling were measured with an eyepiece-micrometer.

The numerical material then underwent statistical interpretation with the calculation of the standard deviation.

After the numerical material from measuring the root lengths was analyzed, a variational series was set up. The entire range of values of a character from minimum to maximum was divided into 16 classes, and the size of the classes or the class interval was 83.5 μ. After the variational series had been set up, histograms were plotted: the incidence of any particular class of roots can be graphically seen.

The cell sizes were determined with intact roots extracted from agar after fixation after Brodskiy or Carnua. The clarification method was used /7/.

The roots were placed in a freshly prepared 5 percent chromic acid solution and left for 24 h. Then dehydration was conducted with solutions of increasingly concentrated ethanol. From absolute ethanol, the roots were transferred into a mixture of methyl ether and ethanol (1:1). The roots were kept in these solutions 10 min, in each case. Finally, the material was placed in pure methyl ether (for 1-2 h), then transferred to a glycerin-ethanol (1:1) mixture.

Description of Experiment, Results, and Discussion

Material in the containers was transported at $4\pm2^{\circ}$ C from the United States to the Soviet Union, where the material was launched 25 Nov 75 on Kosmos 782.

On ground in the Soviet Union was left the container that /5 was subjected to temperature exposures approximating the temperature on board the satellite. At approximately the same periods the eight containers remaining in the United States were installed, four each, at the Ames Research Center and at the university at Stony Brook. These control containers with the

culture they held were for 20 days either in stationary ground conditions (vertical and horizontal variants) or on special installations simulating the absence of gravity (vertical and horizontal clinostats).

The distribution of the experimental variants are in Table 1.

At the end of the space experiment, containers holding the Petri dishes were delivered to the tissue culture and morphogenesis laboratory of the Institute of Plant Physiology, USSR. In Doctor Krikoryan's experiments it was shown that keeping a culture seeded in Petri dishes at 4° C for 7, 14, 21, and 28 days does not change the embryogenic potencies. Only developmental retardation was observed, equal to the period of exposure to the lowered temperature. The containers were breached, visually inspected, and photographed. From each container, dishes Nos. 1, 2, 4, 5, 6, and 8 were shipped to the United States. Dishes Nos. 3, 7, and 9 were left for studies in the Soviet Union.

Study of the structure was conducted on the material of the dishes we obtained, including the dishes (Nos. 3, 7, and 9) sent from the United States on completion of the experiments at Ames and Stony Brook (a total of 33 dishes).

Initial visual inspection showed that in all dishes, independently of the experimental variant, embryoid structures formed (photographs 1 a, b, c). For more detailed microscopic quantitative studies, all structures were divided into several /6 classes, which included:

1) embryoid structures in the "heart" phase

2) embryoid structures in the "torpedo" phase

3) formed embryos with distinct root and cotyledon, with total length from 0.3 mm to 1.5 mm

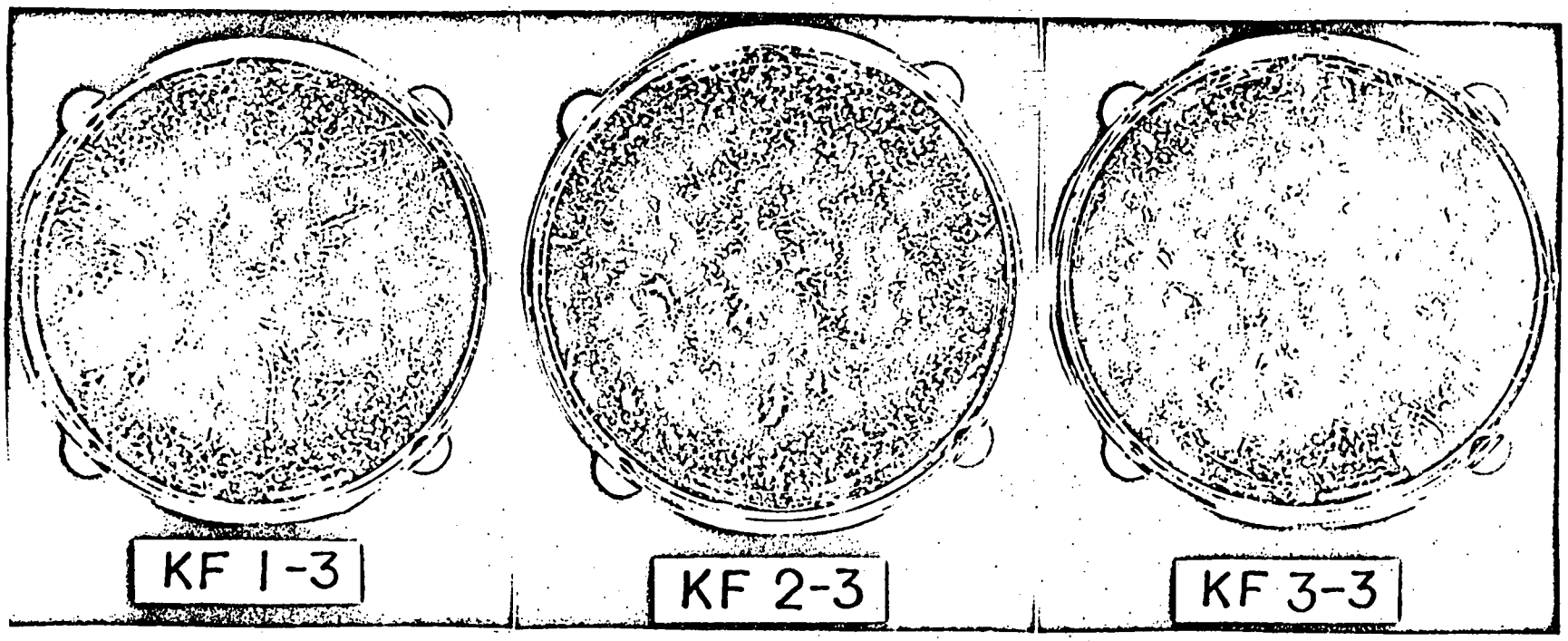
4) seedlings with developed root and developed "above-ground" part, including cotyledon and hypocotyl.

Table 1. Distribution of Experimental Variants

		No
USSR		
Flight 0 g	1 container	KF-1
Flight 1 g	1 container	KF-2
Flight 1 g	1 container	KF-3
United States		
Ames Research Center		
Stationary controls		
Horizontal	1 container	KF-9
Vertical	1 container	KF-7
Clinostat controls		
Horizontal	1 container	KF-10
Vertical	1 container	KF-8
Stony Brook		
Stationary controls		
Horizontal	1 container	KF-13
Vertical	1 container	KF-14
Clinostat controls		
Horizontal	1 container	KF-11
Vertical	1 container	KF-12

Determination of the number of structures by classes showed /8 that during the flight the development of structure during embryogenesis, in the absence of gravity, just as 1 g on the centrifuge, passed through the same stages as on ground. The structures formed reached the final stage (4), both at 0 g and at 1 g in space, as well as at 1 g on Earth.

A

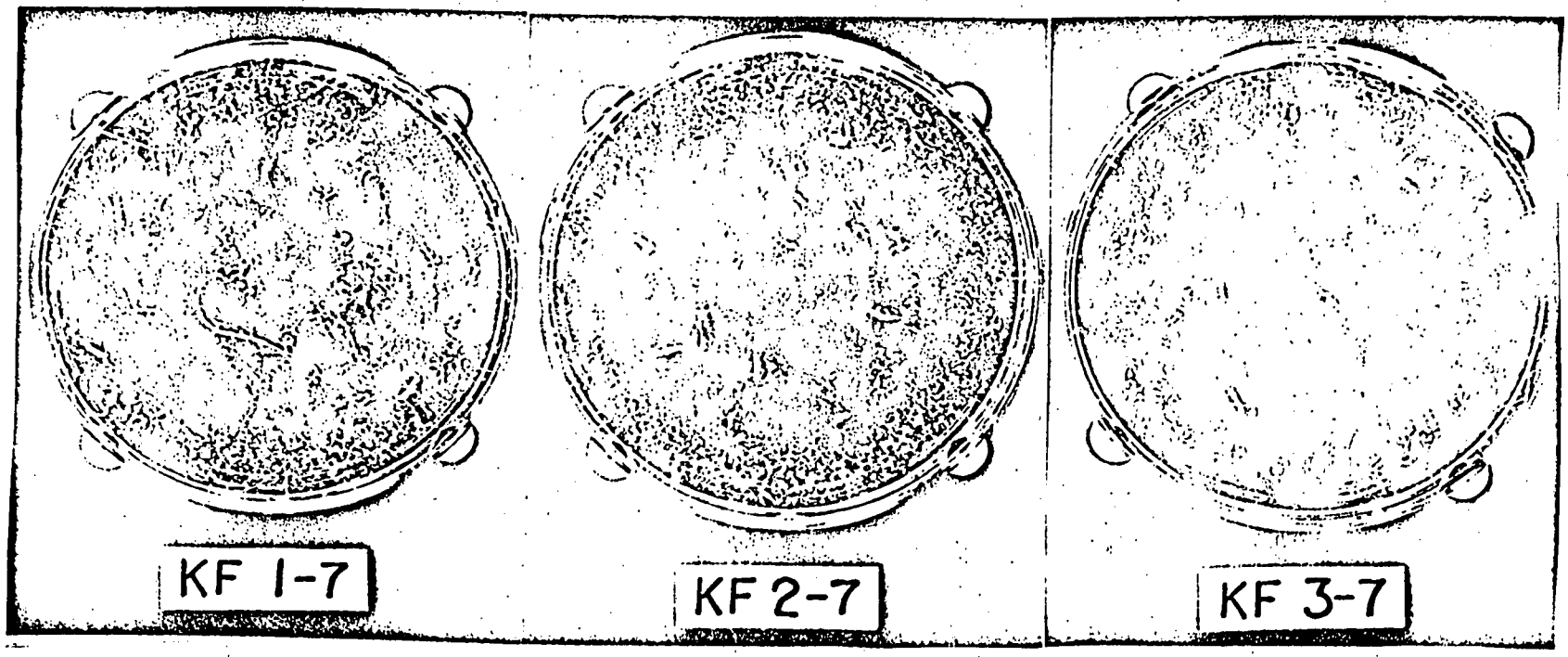


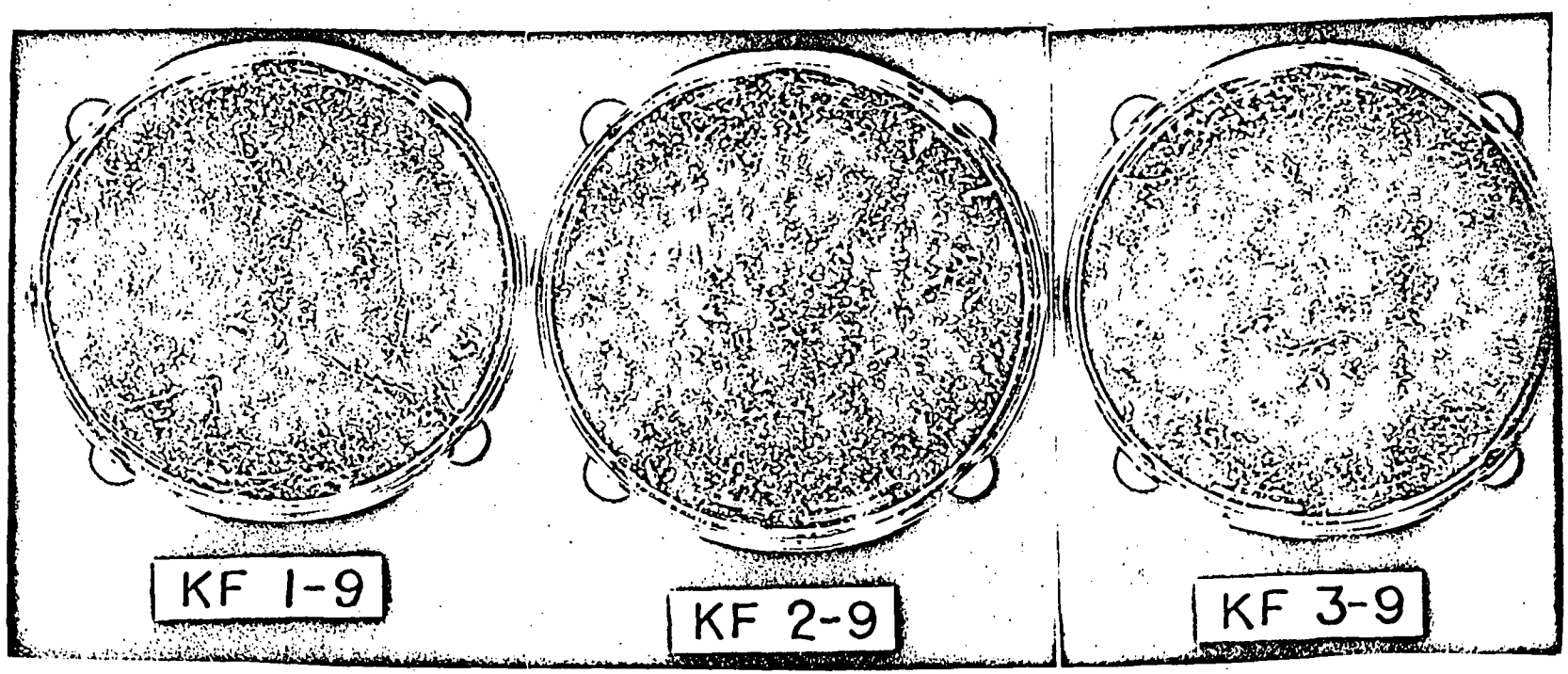
Photograph 1. Cultures containing developing embryonal structures:

in weightlessness (KF-1), in satellite centrifuge (KG-2), and on ground (KF-3)

A. dish No. 3 B. dish No. 7 C. dish No. 9

B




KF 1-9
KF 2-9
KF 3-9

Analysis of the data acquired for all the remaining ground controls also did not disclose a reliable difference between the process happening on Earth in stationary conditions or on various clinostats and the process of embryogenesis in conditions of 0 g on the satellite.

At the same time, we must note the high degree of variations in the given model system; this is expressed in the large values of the standard deviation. With this difference in the process occurring in the same experimental variant, there are no grounds to maintain that any minor difference can be found between variants. Only in the case of total inhibition of the process, and stimulation or retardation of the rate of the process by several times can the role of gravity in this system be disclosed.

The large diversity of structures developing by the 20th day in all experimental variants and the absence of a reliable difference between the process occurring in weightlessness and the process on Earth made unproductive any further comparative study at the microscopic and electron-microscopic levels.

Therefore attention must be given to a certain phenomenon detected in the determination of the size of organs in embryonal structures in stage four. The determination of size revealed /10 that in the case of roots a clear increase in the mean root length is observed for cultures developing at 0 g in space (the length of the mean root is 358 μ compared to 192 μ for the "Earth" variant).

Subsequent work dealt with the causes of longer roots. Here the variational series were set up for three main experimental variants (KF-1, KF-2, and KF-3) for the root lengths; the class size was established and histograms were plotted, reflecting the distribution of roots by classes within each of the three variants (Figure 1).

Table 2. Formation of Embryoid Structures in Different Experimental Conditions. Data correspond to mean number of structures per dish. /9

Experimental Variant	Stage 1 ("heart")	Stage 2 ("torpedo")	Stage 3 ("embryd")	Stage 4 ("seedlings")	Total number of structures
Space 0	I4II±764	78±54	99±50	98±55	I646±744
USSR, Space 1	2363±637	I60±98	I74±70	I78±82	2753±595
USSR, Bubler 1	I430±503	2I7±I27	243±78	I82±86	2058±450
USSR, Stationary, vertical	I875±I023	356±90	225±7I	296±8I	287I±I268
Ames, Clinostat, vertical	II95±754	280±I0I	2I9±200	I87±99	I88I±490
Ames, Stationary, horizontal	2228±II39	272±I56	I87±8I	250±I5I	2934±I038
Ames, Clinostat, horizontal	597±3II	I82±I78	I3I±68	I54±90	II2I±543
Ames, Clinostat, horizontal	I528±I083	I05±I03	4I±35	4I±33	I705±II77
Stony Brook, Clinostat, vertical	I86±74	43±33	I0.7±I0.I	49±3I	288±96
Stony Brook, Stationary, horizontal	987±6II	I09±78	I39.I±I39.I	I3I±I29	I362±602
Stony Brook, Stationary, vertical	I700±760	20[illegible]±78	2I7±84	254±I76	239I±744

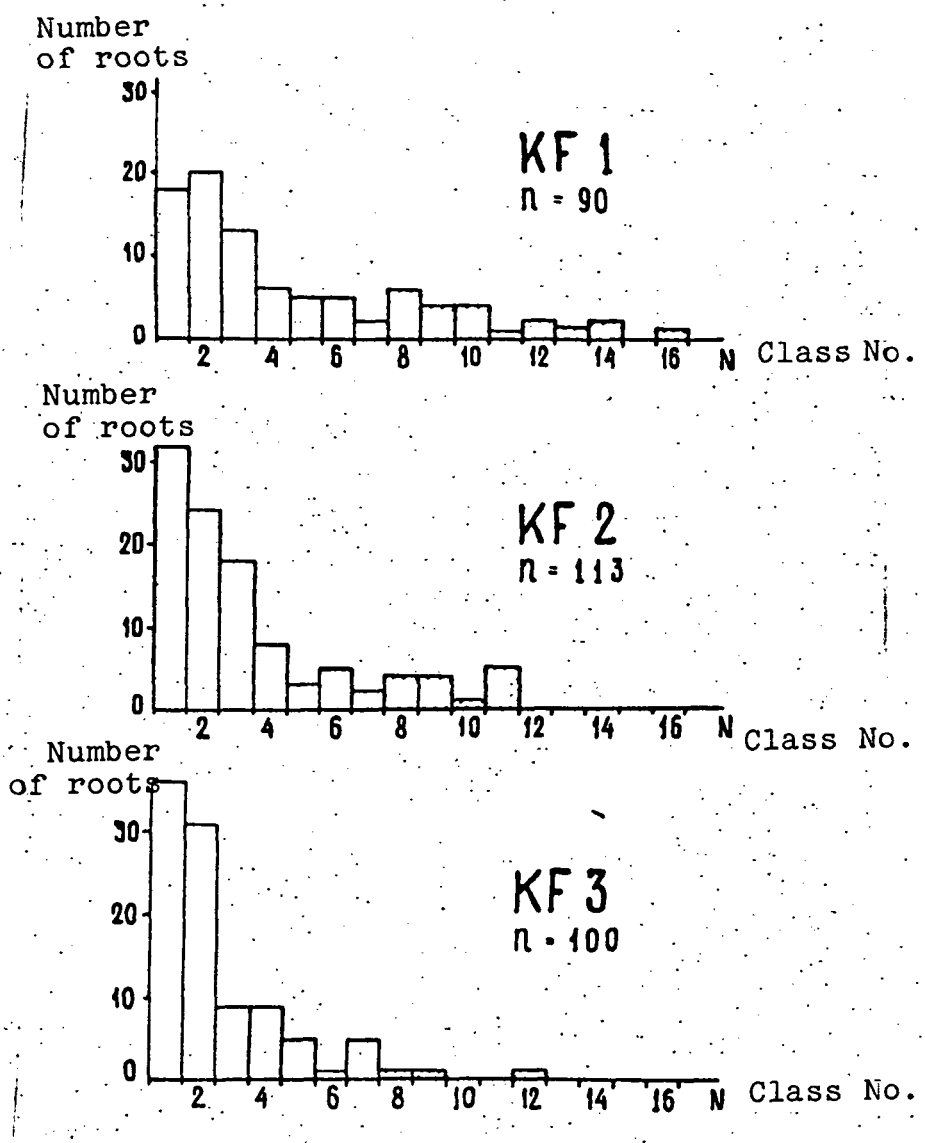


Fig. 1. Distribution of roots by classes
X-axis: class number Y-axis: number of roots within each class

The number of roots in a given class is plotted along the Y-axis. Along the X-axis is plotted the class number; for all three variational series the class size is 83.5 μ.

If we compare the histogram for roots from the KF-1 variant with the histogram for the KF-3 variant, we are struck by the fact that classes Nos. 8-16, corresponding to long roots in the KF-1 variant, are virtually absent in the KF-3 variant. Of the 90 roots measured in the KF-1 variant, 21 roots belong to the long root category, while in the KF-3 variant, only three of the 100 roots measured are long. In other words, among the cultures developing in weightlessness there was found a group exhibiting long roots that were absent in cultures left on Earth in 1 g conditions. The KF-2 variants occupied an intermediate position; for this variant, 14 of the 113 roots measured were long..

The presence of long roots in structures developing in weightlessness can be a consequence of either intensified activity of the root meristem and increased pool of meristematic cells in the meristematic zone, or as a result of the disrupted elongation and enlargement of the root elongation zone. Finally, /12 long roots can belong to embryoid structures that began developing earlier: in this case, long roots much contain a larger number of adult differentiated cells. These possibilities were proven by measurement of cell length by growth zones /8,9/.

Clarified roots after this treatment were ready for examination in a light microscope. For each variant, three roots were processed by the method described. In all variants, roots of average length were used. For KF-1, this consisted of roots with the length 16,000±1632 (μ); in the KF-2 variant, the root length was 5733±944 (μ); and in the KF-3 variant, the root length was 5266±263 (μ). For each root, starting with its tip, three measurements were made at each of 10 separate meristematic cells. The boundary between the meristematic zone and the elongation zone was established from the changes found in the cell size.

Ten separate cells in each field were measured from the established boundary of the meristem in successful fields. The disclosure of cells of constant size (within the limits of at least four to five fields of view) served as a criterion for finding the boundary between the elongation zone and the zone of differentiated cells.

The data are generalized in Table 3. In this table, the figures in columns Nos. 1, 3, 4, 8, and 10 were obtained from microscopic measurements using an eyepiece-micrometer. In this way values were obtained for the length of the meristematic zone (L_M), the elongation zone (L_p), the length of the meristematic cells ($\bar{\ell}_M$), the length of the cells in the elongation zone ($\bar{\ell}_p$), /13 the length of differentiated cells ($\bar{\ell}_g$), and the total root length (L). Data in the columns under Nos. 2, 5, 7, and 9 were obtained by calculations. Examples:

a) determination of the number of cells in one meristematic row N_M

$$N_M = \frac{L_M}{\bar{\ell}_M}$$

b) determination of the number of cells in a single row of the elongation zone N_p

$$N_p = \frac{L_p}{\bar{\ell}_p}$$

As can be seen from this table, the size of the meristematic zone and the size of a single meristematic cells, just as the size of the elongation zone are the same for long roots developing in conditions of weightlessness and for short roots characteristic of structures formed on Earth.

The increased length of roots formed in 0 g conditions is due to the larger number of differentiated cells. It appears that long cells characteristic of cultures developing in weight-

Table 3. Characteristics of Growth Zones of Roots Developing in Weightless (KF-1), on Satellite Centrifuge (KF-2), and on Earth (KF-3)

Variants	Meristem			Elongation zone			Zone of differentiated cells		Total No. of cells in one row	Root Leng
	Zone length /μ/ L_M	Number of cells in one row N_M	Size of cells in one row /μ/ $\bar{l}_M$	Zone length /μ/ L_p	No. of cells in one row N_p	Size of cells in one row /μ/ $\bar{l}_p$	Number of cells in one row N_g	Cell size /μ/ $\bar{l}_g$	$N_K = N_M + N_p + N_g$	L_K
	1	2	3	4	5	6	7	8	9	10
KF-1	126±19	12.3±3.7	10.2±2.7	2640	56	46.8±16.9	185	71.5±12.4	253	1600
KF-2	100±16	11.6±3.7	8.6±2.4	2200	53	41.0±17.0	43	60.5±12.0	107	5733
KF-3	126±9	13.5±4.4	9.3±3.0	2640	61	42.8±13.9	42	58.7±10.7	116	5266

lessness are a consequence of the earlier "wave" of embryogenesis and belong to the more adult embryonic structures. However, it is not precluded that these long roots are a consequence of the increased meristematic activity occurring during some limited period.

But it is hard to answer the question as to when such a burst of meristematic activity can take place, since we do not know anything about the formation of embryoid structures or the developmental dynamics for a given system.

In examining the results from this study, it can be assumed /15 that in this system the determination of cells with respect to their embryonal pathway of development occurred as early as the growing of the suspension culture. This conclusion is suggested by data of other investigators; they indicate that the formation of embryoid structures before the globule stage occurs successfully even in the presence of auxin, and still more so--coconut milk /10/. Therefore, even in this system, where the cells before the launch were seeded on a depleted medium, we can expect an effect on the development of a program that is more determined. Even in this respect, founded on the data obtained, we can make the statement that weightlessness does not affect the actual fact of carrying out the program, though the quantitative aspect (for example, the rate of the process) may have been touched upon. Finding the kind of effect requires a more uniformly reacting model.

At the same time, if we take note of the fact that in 0 g conditions there appeared structures with long roots, we can suggest that weightlessness still caused an earlier (though in a small amount) formation of structures and thus, their correspondingly earlier development.

Conclusions

1. Weightlessness during the flight of Kosmos 782 did not impede embryogenesis in a carrot cell culture. Embryogenesis occurred in weightlessness just as on Earth.

2. The number of embryoid structures and the degree of /16
their differentiation in weightlessness did not reliably differ from the level of differentiation in ground variant structures.

3. The degree of variation in this model system was very high; this was expressed in the large standard deviations and did not permit an estimation of the quantitative difference between experimental variants.

4. In weightlessness, embryoid structures with long roots were found. They belong to classes not found in Earth gravity conditions. And by the size of the growth zones (meristematic zone and elongation zone) of the roots developing in weightlessness did not differ from roots typical of cultures developing on Earth. Roots developing in weightlessness differed only by the increased size of the zone of differentiated cells; this can indicate the earlier development of embryoid structures.

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